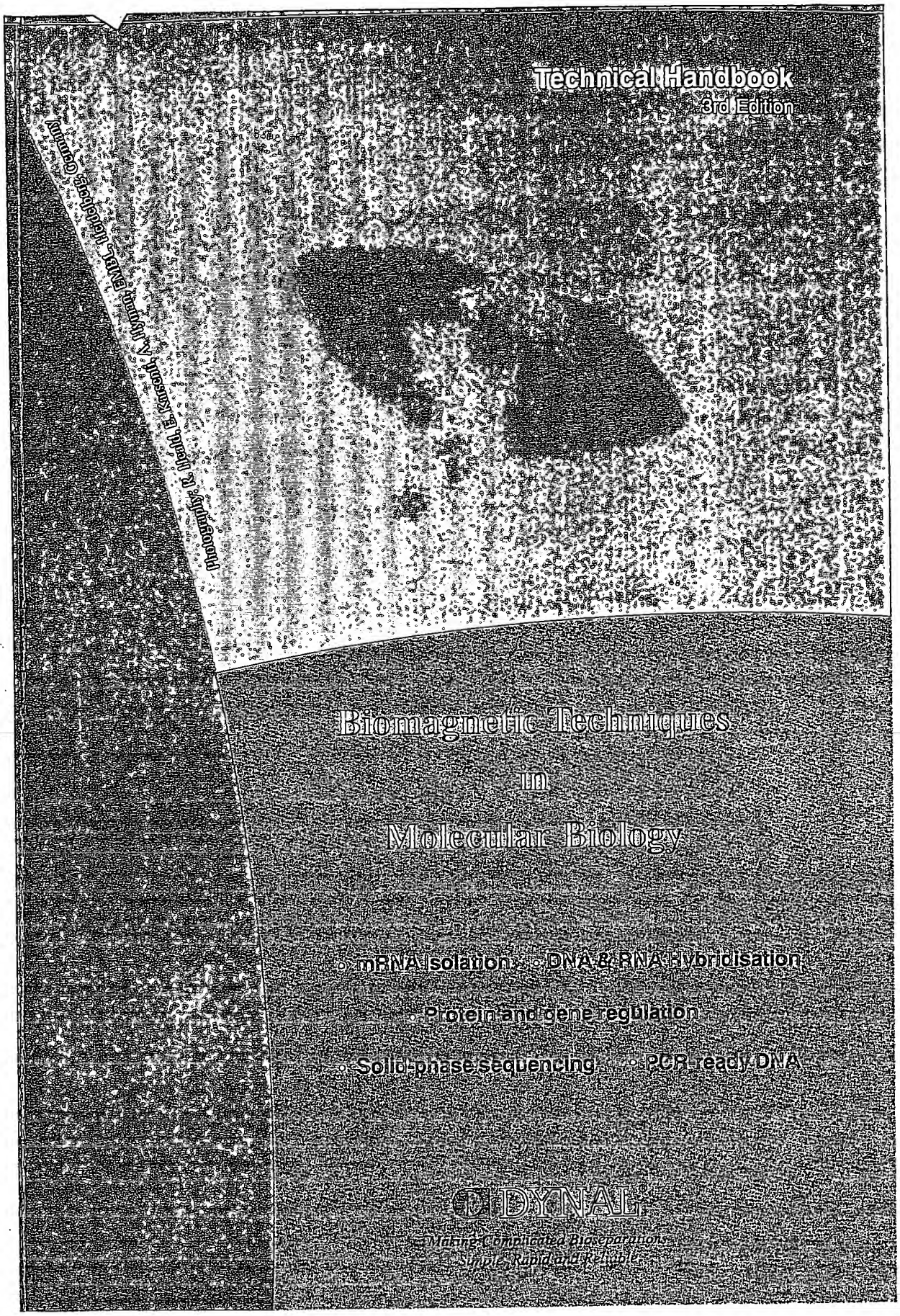
ATTACHMENT 'A'


Technical Handbook
3rd Edition
Biomagnetic Techniques
in
Molecular Biology
• mRNA Isolation • DNA & RNA Hybridisation
• Protein and gene regulation
• Solid-phase sequencing • PCR-ready DNA
DYNAL
Making Complicated Bioseparations
Simple, Rapid and Reliable
Technology A Highly Evolved Company

Amplification and detection

10. Resuspend samples in 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 100 µg gelatin per ml; 0.2 mM (each) dATP, dGTP, dCTP, dUTP; 12.5 pmol of each oligonucleotide primer and 1 U of uracil-N-glycosylase (Gibco BRL, Gaithersburg, Md). Final volume 45 µl.
11. Add *Taq* DNA polymerase (Appligene, Illkirch, France) prior to amplification.
12. Incubate at 37°C for 10 minutes, heat to 95°C for 10 minutes and cool to 80°C in a thermal cycler. Cycling parameters for e.g. IS6110 insertion element were 95°C for 40 sec, 65°C for 40 sec, and 72°C for 15 sec for 50 cycles.
13. Electrophorese on agarose gel and transfer to nylon membranes.
14. Hybridise membranes with ^{32}P-labelled oligonucleotides and detect signals by autoradiography.

1.7 Technical tips

General tips

- DNA fragments longer than 40 base pairs or more complex solutions (e.g. high level of impurities/non-specific DNA) need a longer hybridisation time.
- For larger DNA complexes (>2 kb) use of the Dynabeads kilobaseBINDER™ kit is recommended (please refer to Chapter 5, section 2.4. page 158).
- The tube/tray must be placed on a rotation device/mixer to avoid sedimentation of the Dynabeads.
- Ensure all buffers and equipment are free of RNases when working with RNA.

Blocking repetitive sequences

One major problem of cDNA selection is preventing repetitive sequences present in cDNA and genomic clones from hybridising with each other. This is usually done by pre-annealing either one or both of the sources with various other nucleic acids (e.g. total human DNA or human cot1 DNA). The current protocol recommended uses cot1 DNA to pre-anneal repetitive sequences of the genomic source or the cDNA source (43). A presently unsolved problem is suppression of low copy repeats or repeats that are only present in certain sub-chromosomal regions (156, 157, 158).

Hybridisation and elution

Various mass ratios of cDNA to genomic DNA have been used, ranging from 10,000-fold excess of cDNA to equal amounts of both sources. The eluted cDNAs are subjected to PCR amplification, with the conditions dependent on the primers used. The resultant material is either cycled back for a second or third round of selection and amplification using fresh genomic material, or is cloned directly into phage or plasmid vectors for further analysis.

2. Solid-phase DNA sequencing

2.1 Advantages

Dynabeads Streptavidin can be used to provide a unique solid support for direct sequencing of PCR-amplified cloned and genomic DNA (72). The

main advantages of using Dynabeads Streptavidin and the solid-phase approach are listed below.

- Reproducible DNA sequencing results with high yields are provided.
- The procedure is simple and can be performed in a single tube.
- Time-consuming subcloning, ethanol precipitation, phenol extraction and centrifugation steps are eliminated and the quality of the electrophoretic resolution is improved.
- Efficient production of single-stranded DNA with simultaneous removal of PCR buffers, dNTPs and PCR primers is possible.
- The immobilisation of the PCR product makes it possible to avoid competition between the sequencing primer and the complementary strand of the template that occurs if double-stranded DNA fragments are used directly.
- The presence of the Dynabeads in the sequencing reaction does not inhibit the enzymatic activity of the sequencing enzyme.
- Both strands can be sequenced (bi-directional sequencing).
- The template can be resequenced 3-4 times.
- The system is flexible and is equally useful for both manual and automatic systems.

2.2 The principles of solid-phase DNA sequencing

The solid-phase DNA sequencing scheme first described by Hultman *et al.* (70, 72) is outlined in Figure 6.

First, biotin is introduced into one of the strands of the DNA during the PCR amplification using a primer biotinylated at the 5' end. The PCR product is then immobilised onto the Dynabeads Streptavidin through the biotin/streptavidin interaction. After immobilisation, the Dynabeads/DNA complex is washed extensively to remove all the reaction components resulting from the amplification.

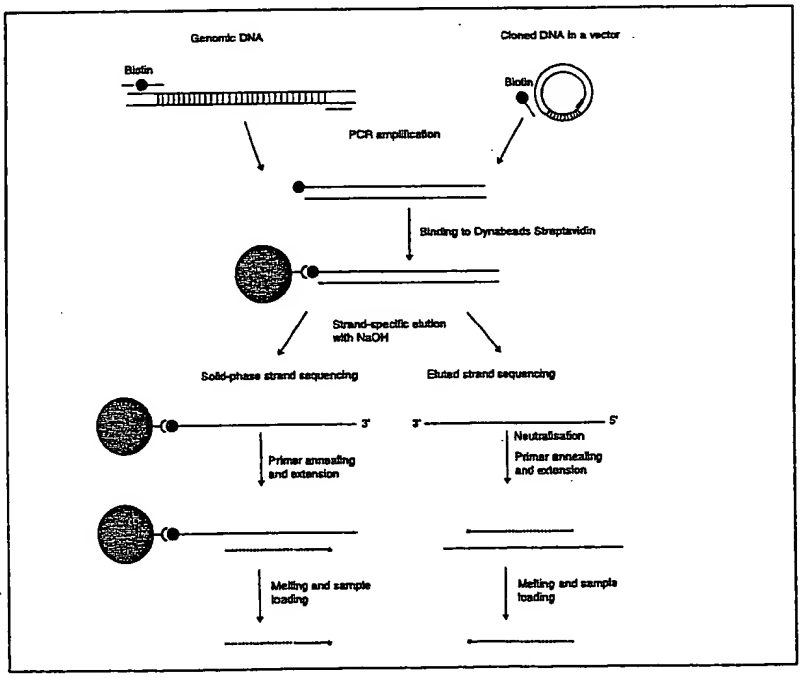


Figure 6. Schematic diagram for solid-phase DNA sequencing directly from cloned and genomic DNA.

The immobilised double-stranded DNA is converted to a single-stranded template by incubation with 0.10 M NaOH for 5 minutes at room temperature and subsequent magnetic separation. Both the immobilised single-stranded template and the eluted strand prepared in this manner are suitable for manual and automated solid-phase DNA sequencing (70, 72) by standard Sanger dideoxy DNA sequencing (143). A 100% single-stranded template is produced, without interference from primers, free nucleotides or the complementary strand.

After the sequencing reaction, the extended material is purified by magnetic separation. It is then separated from the template strand by formamide treatment and loaded directly onto a sequencing gel.

There are numerous examples in which solid-phase DNA sequencing has been demonstrated to be the most reliable tool for DNA analysis, see section 2.6, reference applications (67).

2.3 Materials required (including buffers)

To prepare single-stranded DNA for subsequent solid-phase DNA sequencing, the following material/equipment, including buffers and solutions, will be required:

- Dynabeads M-280 Streptavidin (10 mg/ml) (Product No. 112.05/06)
- Magnet stand, DynaL MPC® (see Chapter 1, section 3)
- Forward and reverse PCR primers (one biotinylated) (see below)
- Sterile water
- A thermal cycler (e.g. GeneAmp® PCR System 9600/9700 or GeneAmp PCR System 2400)
- DNA polymerase
- Toothpicks (for cloned DNA)
- Sterile test tubes, glassware, pipettes
- Tilting/rotation apparatus (e.g. DynaL mixer)
- Any DNA sequencing kit for standard Sanger sequencing
- Internal sequencing primers

Buffers and solutions

- **Binding & Washing (B&W) buffer**

A buffer with a final salt concentration of at least 1.0 M is recommended for optimal binding of the DNA template. A suggested buffer follows:

Binding & Washing buffer (2x concentration)	10 mM Tris-HCl, pH 7.5 1.0 mM EDTA 2.0 M NaCl

Note: A binding buffer with 3 M LiCl instead of 1 M NaCl has been reported to improve binding of large DNA fragments (> 1 Kb) (67). LiCl is substituted for NaCl because LiCl has a higher solubility than NaCl (NaCl tends to precipitate at high concentrations) (See Chapter 1, section 2.3, page 156 for optimal binding conditions for DNA fragments of different sizes). For binding of fragments < 2 Kb, use of the Dynabeads KilobaseBINDER™ kit is recommended (see Chapter 1, section 2.4, page 158).

• PCR buffer	20 mM Tris-HCl, pH 8.5 at 20°C 50 mM KCl 0.1 % Tween 20 2.0 mM $MgCl_2$ 200 µM of each dNTP
• TE buffer	10 mM Tris-HCl, pH 7.5 1 mM EDTA
• Melting solution	fresh 0.10 M NaOH
• Neutralising solution	0.200 M HCl or 1 M Tris-HCl
• TT buffer (for resequencing templates)	10 mM Tris-HCl, pH 8.0 0.1 % Tween 20

PCR primers

Each PCR amplification should be performed with a primer set that consists of one biotinylated and one non-biotinylated primer. Biotinylation of oligonucleotide primers at the 5' end is recommended. Biotinylation of oligonucleotide primers is described in Chapter 5, section 5.1. page 166. Purify the biotinylated oligonucleotide by reverse HPLC following the manufacturer's instructions.

For the amplification of **genomic DNA**, specific biotinylated primers for the target gene/sequence must be designed and synthesised.

For the amplification of **cloned DNA**, biotinylated and non-biotinylated versions of the following forward and reverse PCR primers are recommended:

forward: 5'-CGCCAGGGTTTTCCCAGTCACGACG-3'
reverse: 5'-GCTTCCGGCTCGTATGTTGTGTGG-3'

These PCR primers are complementary to conserved sequences upstream and downstream of the multiple cloning site (MCS) in common cloning vectors (e.g. pUC, M13 and pBluescript®).

λgt11 is not a suitable vector for use with the recommended forward and reverse PCR primers.

Table 3 shows the locations of forward and reverse PCR primers as well as common sequencing primer priming sites on current cloning vectors. These vectors contain *E.coli* β-galactosidase sequences used for blue/white colour selection.

Blue/white colour selection: Cloning vectors containing the portion of the lacZ gene providing α-complementation can be used. When a cloned insert interrupts the lacZ α-peptide in the multiple cloning site (MCS), no complementation occurs and transfected or transformed cell colonies appear white when plated on indicator plates.

Sequencing primers

For sequencing of genomic DNA, specific sequencing primers for the amplified target gene/sequence must be designed and synthesised. It is recommended to use different internal sequencing primers from the PCR primers.

There are many commercially produced primers for sequencing of cloned DNA. The most common primers available are for the M13mp/pUC vectors, but these primers are also suitable for the other vectors indicated in Table 3. In this table the following forward and reverse sequencing primer are used as examples:

Forward Sequencing Primer, M13/pUC: 5'-GTTTTCCCAGTCACGAC-3'
(-40, 17-mer)
Reverse Sequencing Primer, M13/pUC: 5'-CAGGAAACAGCTATGAC-3'
(-29, 17-mer)

If cycle sequencing is being performed, the non-biotinylated version of the recommended forward and reverse PCR primers can be used as sequencing primers (8).

Table 3: Binding regions at different cloning vectors for recommended PCR primers and sequencing primers.

Cloning vector	Size	Binding region* for forward PCR primer	Binding region for forward sequencing primer	Multiple cloning sites (MCS)	Binding region for reverse sequencing primer	Binding region for reverse PCR primer	Size of the PCR product without any insert
pUC7 [a,b,c]	2674 bp	352-376	359-375	397-442	469-453	519-496	168 bp
pUC8	2665 bp	352-376	359-375	397-433	460-444	510-487	159 bp
pUC9	2665 bp	352-376	359-375	397-433	460-444	510-487	159 bp
pUC12	2680 bp	352-376	359-375	397-448	475-459	525-502	174 bp
pUC13	2680 bp	352-376	359-375	397-448	475-459	525-502	174 bp
pUC18	2686 bp	352-376	359-375	397-454	481-465	531-508	180 bp
pUC19	2686 bp	352-376	359-375	397-454	481-465	531-508	180 bp
pUC118	3.2 kb	352-376	359-375	397-454	481-465	531-508	180 bp
pUC119	3.2 kb	352-376	359-375	397-454	481-465	531-508	180 bp
M13mp7 [a,b,c,d]	7237 bp	6321-6297	6314-6298	6231-6276	6204-6220	6154-6177	168 bp
M13mp8	7228 bp	6312-6288	6305-6289	6231-6276	6204-6220	6154-6177	159 bp
M13mp9	7228 bp	6312-6288	6305-6289	6231-6276	6204-6220	6154-6177	159 bp
M13mp10	7243 bp	6327-6303	6320-6304	6231-6282	6204-6220	6154-6177	174 bp
M13mp11	7243 bp	6327-6303	6320-6304	6231-6282	6204-6220	6154-6177	174 bp
M13mp18	7249 bp	6333-6309	6326-6310	6231-6288	6204-6220	6154-6177	180 bp
M13mp19	7249 bp	6333-6309	6326-6310	6231-6288	6204-6220	6154-6177	180 bp
pBluescript® SK (+/-) [b,e,f]	2958 bp	576-600	583-599	657-759	828-812	878-855	303 bp
pBluescript® II SK (+/-)	2961 bp	573-597	580-596	657-759	828-812	878-855	306 bp
pBluescript® II KS (+/-)	2961 bp	573-597	580-596	657-759	828-812	878-855	306 bp
Lambda ZAP®II [b,j]	40.82 kb	same distances as for pBluescript® SK (-)					303 bp
pGEM®-3Z [b,g,h]	2743 bp	2679-2703	2686-2702	5-61	128-112	178-155	243 bp
pGEM®-4Z	2746 bp	2677-2701	2684-2700	7-63	126-110	176-153	243 bp
pGEM®-3Zf(+/-)	3199 bp	3133-3157	3140-3156	5-61	120-104	170-147	237 bp
pGEM®-5Zf(+/-)	3003 bp	2937-2961	2944-2960	10-113	177-161	227-204	294 bp
pGEM®-7Zf(+/-)	3000 bp	2934-2958	2941-2957	10-110	174-158	224-201	291 bp
pGEM®-9Zf(-)	2925 bp	2837-2861	2844-2860	5-54	119-103	169-146	258 bp
pGEM®-11Zf(+/-)	3223 bp	3157-3181	3164-3180	19-85	144-128	194-171	261 bp
pGEM®-13Zf(+)	3181 bp	3115-3139	3122-3138	11-44	144-128	152-129	219 bp
pCR® II [b,**]	3932 bp	457-433	450-434	269-381	205-221	155-178	303 bp

*) The binding region is given in the 5'-3' direction.
**) The pCR II is identical to the TA Cloning® Vector designed for direct cloning of PCR products manufactured by Invitrogen Corporation.

a. Yanisch-Perron C, Vieira J, Messing J. (1985). Improved M13 phage cloning vectors and host strains; nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33 103-119.
b. Sambrook J, Fritsch EF, Maniatis T. (1989). Molecular cloning; A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Book 1, 1.13 - 1.20, 2.52-2.53, 4.7-4.12.
c. Amersham Pharmacia Biotech. Biodirectory 1998, pp 93-94.
d. New England Biolabs, 1996/1997 Catalog, pp. 201-203.
e. Nucleotide sequence and restriction sites of pBluescript® II SK (+), Stratagene® Cloning Systems.
f. Stratagene® Cloning Systems, 1997/87 Product Catalog, pp.19, 38-39.
g. Promega Corporation, Technical Bulletin 150:6/92 pp. 7-8.
h. Promega Protocols and Applications Guide, Third Edition, 1996, pp. 386-389.
i. Invitrogen Catalog 1998, Molecular Biology Solutions pp.65,155.

2.4 Protocols

2.4.1 Template preparation

This protocol is used for preparing immobilised PCR-amplified single-stranded templates for subsequent solid-phase DNA sequencing from cloned and genomic DNA samples.

1. Preparing the Dynabeads Streptavidin

a. Wash **5 - 20 µl** (depending on sequencing chemistry) of Dynabeads Streptavidin with **20 µl** of 2 x Binding and Washing (B&W) buffer. The Dynabeads may be washed in bulk; simply multiply the volumes written in boldtype with the number of sequencing reactions.

Note: T7 DNA polymerase (Sequenase®) chemistry

- use 20 µl (200 µg) of Dynabeads per DNA template.

***Taq* cycle sequencing chemistry**

- use 10 µl (100 µg) of Dynabeads per DNA template > 250 base pairs.
- use 5 µl (50 µg) of Dynabeads per DNA template < 250 base pairs.

AmpliTaq® FS and ThermoSequenase chemistry

- requires less than half the amount of template used with Sequenase sequencing chemistry.

b. Resuspend the Dynabeads in 40 µl of 2 x B&W buffer.

2. Direct isolation of DNA from bacterial colonies

a. Pick a fresh, single bacterial colony (plaque) of the appropriate *E.coli* host containing the inserted plasmid/phage to be sequenced, from an agar plate using a sterilised tip or toothpick.

b. Suspend the colony in 10 µl PCR buffer in a PCR tube.
Note: A lysis step is unnecessary as bacteria will lyse during the first PCR step.

3. PCR amplification guidelines

a. Use up to 0.5 µg of **genomic DNA** and 5-10 pmol of each specific flanking primer (one biotinylated and one non-biotinylated) in the PCR reaction. For **cloned DNA**, use approximately 5-10 ng plasmid/phage DNA in 1-2 µl TE buffer.
Note: It is important to use only the recommended amount of PCR primer, as an excess of unincorporated biotinylated primer will compete with biotinylated PCR products and decrease amount of amplified product bound to the bead surface. The recommended primers for amplification of cloned DNA are designed for use at high annealing temperatures. A 25 cycle PCR programme with denaturation for 30 seconds at 96°C, annealing for 1 minute at 65°C and extension for 2 minutes at 72°C is recommended. See also point d below.

b. A 3-fold excess of non-biotinylated primer may be used to help drive complete extension of the biotinylated primer. This will further reduce the possibility of unincorporated biotinylated primer interfering with sequencing (8, 112).

c. Use highly purified biotinylated primers in the PCR amplification (see Chapter 5, section 5.1. page 166) to ensure a PCR product which has a high biotin content.

d. To prevent structure-specific endonuclease activity by *Taq* DNA polymerase that may cleave single-stranded DNA and remove the 5' biotin from the primers, complete the PCR amplification with extension at 72°C for 5 minutes. This step will also increase the amount of full length products by ensuring complete extension of incomplete products from previous cycles.
Note: In certain clinical applications, target DNA may be present in very low concentrations or in a relatively impure sample (33). Both of these problems may occur simultaneously (3). In such cases, the desired products can be generated with increased specificity by using a nested PCR procedure (a two-step PCR with first an outer primer set and secondly an inner primer set where one of the two primers is biotinylated).

Note: Secondary structures in the target DNA may sometimes hinder the extension by polymerase. Please refer to section 2.5 for technical tips.

4. Immobilisation of the PCR product

a. Add 40 µl amplified PCR product to 40 µl 2 x B&W buffer containing the prewashed Dynabeads Streptavidin.
b. Incubate for 15 minutes at room temperature keeping Dynabeads suspended by gently tipping tube or plate.
   Note: For DNA fragments >1kb, incubate for 15-60 minutes at 43°C (67). To decrease steric hindrance when immobilising long PCR products (>1kb), add 50% more Dynabeads or use the Dynabeads kilobaseBINDER™ kit system (please refer to Chapter 5, section 2.4, page 158).

5. Melting the DNA duplex

a. Place tube/plate containing immobilised product in a suitable magnet stand (Dynal MPC) and remove supernatant with a pipette.
b. Remove tube/plate from magnet stand and add 40 µl of 2 x B&W buffer to wash the Dynabeads/nucleic acid complex.
   Note: The immobilised product can now be stored at 2-8°C for several weeks.
c. Repeat step a.
d. If you only wish to work with solid-phase template, resuspend Dynabeads/nucleic acid complex in freshly prepared 0.1 M NaOH solution (50 µl).
   Note: If you wish to recover the non-biotinylated strand for DNA sequencing, use exactly 8 µl 0.100 M NaOH. Use a 1.000±0.005 M volumetric solution of NaOH and dilute this to 0.100 M. Aliquot and store the NaOH at -20°C for not more than 3 months. Use either freshly prepared NaOH solution or NaOH that has been prepared and stored as above.
e. Incubate at room temperature for 5 minutes.

6. Separating the DNA strands

a. Place the tube/plate into the magnet stand and transfer the NaOH supernatant to a clean tube/well. The non-biotinylated single-stranded DNA can now be sequenced (see section 2.4.3).
b. Wash the Dynabeads (with the immobilised biotinylated strand)
   - once with 50 µl 0.1 M NaOH
   - once with 50 µl of 1 x B&W buffer
   - once with 50 µl TE buffer

   Place tube/plate in magnet stand when washings are being removed.
   Note: All resuspensions should be made by gentle pipetting or tapping so that any possible aggregates of Dynabeads complexes are suspended.
c. Remove supernatant and adjust volume with water, according to template volume of sequencing protocol.

2.4.2 Solid-phase strand sequencing

All common sequencing kits for manual and automated dideoxy sequencing procedures can be used. Solid-phase sequencing can be performed with all DNA polymerases suitable for sequencing, such as T7 (Sequenase), Klenow, *Taq*, *Tth* and *Bst*.

a. Add Dynabeads complex with immobilised single-stranded template to annealing mixture containing appropriate sequencing primer. Handle Dynabeads with immobilised ssDNA as if it were a conventional DNA template.

Note: For genomic sequencing, internal sequencing primers are recommended. Do not employ the same primers in the sequencing reaction as used for generating the PCR product. If possible use internal sequencing primers.

b. Continue sequencing reaction according to your own sequencing protocol (annealing and labelling reactions).
c. For optimal results, after extension is completed, remove supernatant containing excess sequencing primers and unincorporated nucleotides.
   Note: Elimination of glycerol and salts from products of DNA sequencing reactions increases the quality of sequence data (i.e. band intensity, resolution, and base calling accuracy) (164).
d. The newly synthesised strands (termination products) can be eluted off by adding 95% formamide solution and incubating for 2 minutes in a 37°C water bath.
   Note: If the immobilised template is not to be regenerated, both strands can be eluted off by adding 95% formamide and incubating at 95°C for 3 min. These conditions will destroy the interaction between streptavidin and biotin and release the DNA templates together with dideoxy fragments.

Note:
For manual sequencing:
Sequenase
Rinse the Dynabeads with B&W buffer or 70% EtOH or follow the same procedure as for Sequenase Dye Primer.

For automated sequencing:
Sequenase Dye Primer
Rinse the Dynabeads once in TE buffer to stop the reaction and remove excess primers in loading buffer.

Sequenase Dye Terminator
Rinse the Dynabeads 2-4 times in 0.01 M Tris-HCl, pH 8.0, 0.1% Tween-20 to remove unincorporated dyes. Resuspend in loading buffer or 70% EtOH.

***Taq* Dye Primer**
Pool the reactions and ethanol precipitate or use spin columns (Centri-Sep®). Resuspend in loading buffer.

***Taq* Dye Terminator**
Excess dye terminators need to be removed. Dynal recommend to use biotinylated sequencing primers to clean up the sequencing product (section 2.4.4).

e. Collect the Dynabeads on the tube wall with the magnet stand and remove the supernatant containing the dideoxy fragments.
   Note: This step may be omitted. The extension sequencing mixture and Dynabeads in formamide solution can be loaded directly onto the sequencing gel without distorting the migration of dideoxy fragments.
f. Load each reaction directly onto the sequencing gel.

Reconditioning of template
a. Resuspend Dynabeads-bound template in 0.1 M NaOH, 0.1% Tween.
b. Wash once in B&W buffer, 0.1% Tween and resuspend in same solution again.
   Note: Template may be stored in this buffer at 4°C until required for resequencing.

2.4.3 Eluted strand sequencing

The non-biotinylated strand removed during the alkali denaturation step may also be recovered for DNA sequencing. Several successful methods have been reported for the preparation of the eluted, non-biotinylated ssDNA remaining after solid-phase DNA sequencing (70, 86, 98, 131, 160). In one of these, the immobilised strand was sequenced by a Sequenase-based method and the eluted, free strand was successfully sequenced by *Taq* cycle sequencing (131).

In some cases it is difficult to sequence the eluted strand because of problems in adjusting either the pH or the ionic strength in the neutralisation step. A method for concentrating the eluted non-biotinylated strand using Dynabeads and magnetic separation has been developed. This is achieved by labelling the non-biotinylated PCR primer with digoxigenin and then using Dynabeads coated with anti-digoxigenin antibodies to capture the eluted strand, after alkali treatment (17). The scheme for solid-phase sequencing of both strands of PCR amplified DNA using Dynabeads Streptavidin and Dynabeads coated with anti-digoxigenin is outlined in Figure 7.

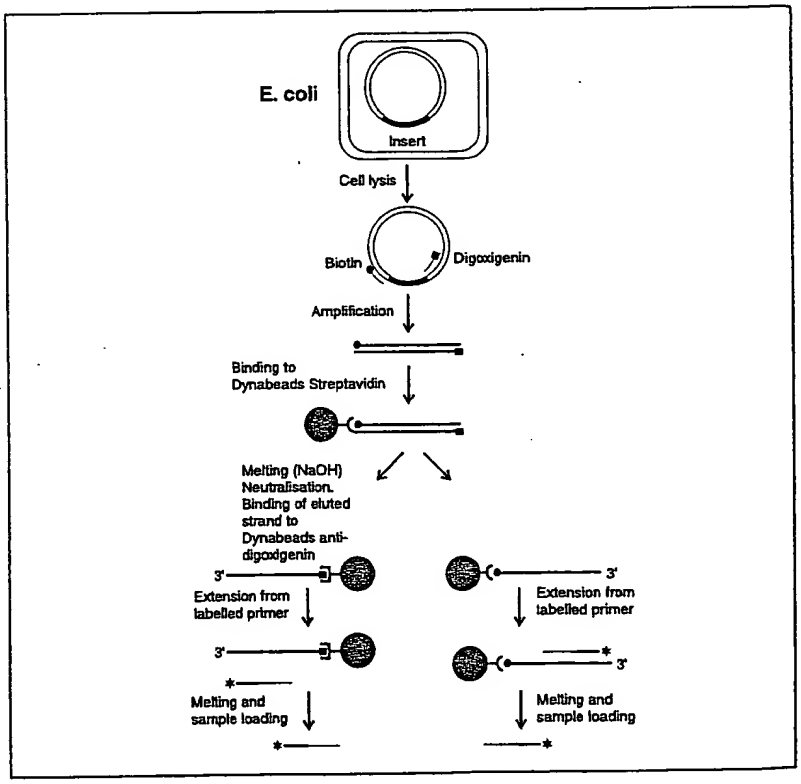


Figure 7. Schematic diagram of solid-phase sequencing using Dynabeads Streptavidin and Dynabeads coated with anti-digoxigenin.

Protocol for solid-phase sequencing of both strands of PCR amplified DNA with Dynabeads Streptavidin and Dynabeads coated with anti-digoxigenin

1. Preparation of Dynabeads Streptavidin.
   Wash 20 µl (200 µg) Dynabeads Streptavidin in 20 µl 2 x B&W buffer and then resuspend in 40 µl 2 x B&W buffer.
2. Preparation of anti-digoxigenin coated Dynabeads M-280 Sheep anti-Mouse IgG (Prod. No. 112.01/02).

a. Wash the Dynabeads once in coating buffer (0.0075 M PBS, pH 7.4).
b. Add antibodies (3.4 µg antibodies/mg Dynabeads used at a concentration of 100 mg Dynabeads per ml coating buffer) and rotate at 4°C.
c. Wash the now coated Dynabeads twice with PBS-buffer containing 0.1% BSA and store in PBS-buffer containing 0.1% BSA and 0.02% NaN_3.

3. PCR conditions.
   a. 5 pmol of each labelled primer (carrying either biotin or digoxigenin).
   b. Vector pGA7.3.
   c. The Perkin Elmer Cetus AmpliTaq kit.
   d. PCR cycles of 96°C for 30 seconds, 65°C for 1 minute and 72°C for 2 minutes. A volume of 50 µl.
   e. A hybrid thermal cycler can be used.
4. Immobilisation of the PCR product.
   Add 40 µl of the amplified PCR product to 40 µl of the prewashed Dynabeads Streptavidin (step 1) and incubate at room temperature for 15 minutes, ensuring that the Dynabeads remain in suspension.
5. Melting the DNA duplex.
   Wash the Dynabeads with the immobilised PCR product once in 40 µl 2 x B&W buffer. After removing the supernatant, add 8 µl 0.1 M NaOH and incubate at room temperature for 10 minutes.
6. Separating the DNA strand.
   a. Use a magnet stand (Dynal MPC-E) to collect the Dynabeads and transfer the supernatant containing the eluted strand to a new tube and add 8 µl 0.1 M NaOH.
   b. Wash the Dynabeads with the immobilised biotinylated strand once with 50 µl 0.1 M NaOH, once with 50 µl B&W buffer and once with 50 µl TE buffer. Remove the supernatant and adjust the volume with distilled water according to the template volume in the sequencing protocol.
7. Immobilising the digoxigenin labelled strand.
   a. Neutralise the supernatant from step 6 a (2 x 8 µl) with an equal volume of 0.1 M HCl and 2 µl 1M Tris-HCl, pH 7.4. Adjust the volume to 50 µl with PBS, pH 7.4.
   b. Wash 500 µg Dynabeads anti-digoxigenin (step 2) once in PBS and resupend in 50 µl PBS before adding to the neutralised digoxigenin labelled strand solution.
   c. Incubate the mixture at room temperature for 30 minutes with occasional mixing. After washing once with 100 µl PBS and 50 µl 1 x TE buffer, resuspend the Dynabeads in water, according to the template in the sequencing reaction.
8. Sequencing reaction.
   Follow the manufacturer's recommended protocol.
   Annealing mixture:
   a. Add 1 µl reverse sequencing primer and 2 µl sequencing buffer to 7 µl biotin labelled strand immobilised on Dynabeads Streptavidin (200 µg). Heat for 2 minutes at 65°C and then slowly cool to 35°C to anneal.
   b. Add 7 µl Dynabeads anti-digoxigenin carrying immobilised digoxigenin labelled primer to 1 µl forward sequencing primer and 2 µl sequencing buffer. Heat for 2 minutes at 65°C and then slowly cool to 35°C to anneal.

   For the remainder of the protocol follow the manufacturer's instructions (please refer to section 2.4.2).

After completion of reaction, heat the samples to 75°C for 2 minutes immediately before loading to elute off the synthesized strand. After magnetic separation, load 4 µl of each reaction onto a sequencing gel.

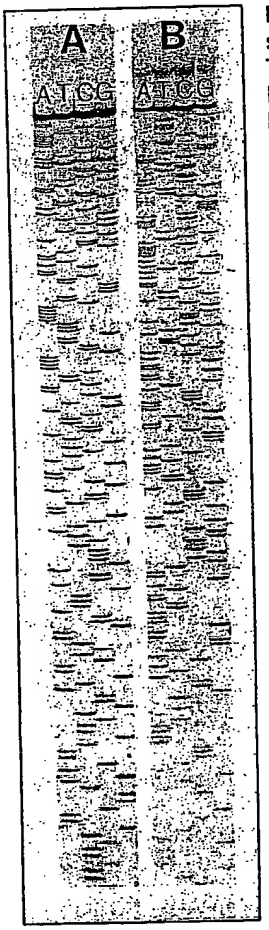


Figure 8. Autoradiograms of sequencing gels of the solid-phase strand (A) and also the eluted, complementary DNA strand (B) (86).

Protocols for different neutralisation procedures (eluted strand sequencing) - alternatives to digoxigenin labelling
To recover and determine the nucleotide sequence of the complementary DNA template in the alkali supernatant, the following four procedures have given successful results. Choice of procedure is dependent upon application.

1. **Neutralisation with HCl**
   Neutralise alkali supernatant with precisely 4 µl 0.200 M HCl* and 1 µl 1.0 M Tris-HCl (pH adjusted to optimum of sequencing enzyme) (70). Immediately mix with a pipette and adjust volume with water according to sequencing protocol.
   Note: Always use the same pipette for both NaOH and HCl, as small differences in calibration between different pipettes can cause neutralisation problems.
   *Use a 1.000±0.005 M volumetric solution of HCl and dilute this to 0.200 M. Aliquot and store the HCl at -20°C for not more than 3 months.
2. **Neutralisation with ammonium acetate**
   Elute non-biotinylated ssDNA from pelleted Dynabeads as follows:
   Add 300 µl of 0.15 M NaOH and incubate for 5 minutes.
   Place tube in magnet stand (Dynal MPC) and transfer supernatant to a new tube.
   Precipitate non-biotinylated ssDNA as follows:
   Neutralise by adding 150 µl of 5 M ammonium acetate, pH 6.6.
   Perform isopropanol precipitation using 2 µg of nucleic acid free glycogen as a co-precipitant (98).
3. **Ethanol precipitation**
   Recover the unbiotinylated strands as follows:
   Add 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol to NaOH washes. Incubate at -20°C for 2 hours. Centrifuge samples at 12,000 g for 10 minutes and wash pellets twice with 70% ethanol, vacuum-dry and redissolve in 5 µl of water (160).
4. **Centricon®-30 microconcentrator**
   An alternative method (86) avoids the requirement for accurate neutralisation and precipitation of the alkali supernatant by using a Centricon-30 microconcentrator (Figure 8).

2.4.4 Solid-phase clean-up of dye-terminator biotinylated sequencing products

In Dye terminator sequencing, any molecule that unspecifically primes DNA polymerisation reactions will add to the instrumental background noise. This is because all the terminated fragments, specific and unspecific, will carry a fluorescent label. This may result in poor sequencing ladders with a high background of unspecific sequencing fragments. Successful direct cycle sequencing is thus dependent upon totally optimised PCR reactions with new sequencing enzymes purified by spin column, ethanol precipitation or enzymatic removal of primers and other single-stranded DNA fragments.

Using Dynabeads Streptavidin for downstream purification of sequencing products after completed dye terminator sequencing reactions, eliminates the requirement for spin columns, centrifugation or precipitation cleanup procedures. The cleanup procedure using Dynabeads Streptavidin takes

less than ten minutes, saving both time and money, and can be fully automated by using, for example, Biomek2000 Laboratory Automation Workstations (Beckman Instr., Inc., Fullerton, USA), equipped with a magnet station (MPC-auto96) with suitable lifts.

Template preparation

1. Run a standard PCR reaction with non-biotinylated primers to amplify genomic DNA sample.
   Note: The amount of PCR product added to the sequencing reaction may have to be optimised for specific template. For longer templates, the ratio of ddNTP: dNTP may have to be decreased to create more longer fragments. This is because short fragments will bind faster and Dynabeads may become saturated with short fragments.

Cycle sequencing

2. Run standard Dye terminator sequencing reactions (ABI PRISM™ Dye Terminator Sequencing kit with AmpliTaq FS) with biotinylated sequencing primers and preferably internal primers also (112).
3. For each sequencing reaction wash 50 µg (5 µl) Dynabeads Streptavidin once in 20 µl 2 x B&W buffer and resuspend Dynabeads in same volume (20 µl) B&W buffer.
4. To every 20 µl sequencing reaction, add 20 µl resuspended Dynabeads Streptavidin.
5. Incubate at room temperature (15-25°C) for 2-5 minutes.
6. Place sample tube in magnet stand (Dynal MPC) and remove the supernatant.
7. Remove tube from magnet stand and wash Dynabeads complex once in 50 µl 70% ethanol by pipetting up and down five to ten times until the pellet is properly resolved. Avoid air bubbles.
8. Replace tube in the magnet stand and leave for 1 minute before removing as much ethanol as possible. Any residual ethanol may interfere with gel mobility.
9. For each reaction add 5 - 10 µl loading buffer to sequencing reaction.
10. Incubate at 90°C for 2 minutes.
11. Place tube in magnet stand. Remove supernatant containing eluted fragments and load supernatant directly onto a pre-run sequencing gel.
    Note: Biotinylated sequencing products will move through the gel more slowly than non-biotinylated sequencing products.

2.5 Technical tips and troubleshooting

General tips

Solid-phase DNA sequencing with Dynabeads Streptavidin is the most reliable method for directly sequencing PCR products and usually autoradiograph or fluorogram banding patterns are excellent and clear. However, unsatisfactory results may occasionally be obtained. If this happens, it is important that the result is evaluated and its appearance used to deduce the most probable cause of the problems. In Brown (17) actual experiences in attempting to understand the procedures when the author first sequenced DNA are described. This book also includes a troubleshooting guide for manual sequencing.

In general, each series of sequencing reactions should include one control reaction using high quality DNA. For single-stranded DNA, the use of 1 µg commercially available M13mp18 DNA is recommended.

Problems arising during the PCR amplification may also affect sequencing data. The most common effect is related to PCR artefacts (e.g. truncated products) during the PCR amplification due to secondary structures (hair-pin loops). This problem can be overcome by performing a hot start PCR, adding DMSO (5% or 10% final concentration) to the PCR reaction mixture or increasing the annealing temperature in the PCR by 2-3°C.

Biotinylation
All biotin reagents should contain a spacer arm, at least a 6-carbon linker, to reduce steric hindrance. Reverse-phase HPLC or FPLC® purified biotinylated primers are recommended. See Chapter 5, section 5.1, page 166 for detailed protocols.

Secondary structure
The inclusion of additives, such as dimethyl sulphoxide (DMSO) and glycerol in the reaction buffer may allow amplification of DNAs with complex secondary structures (148). DMSO can be added to the reaction to a final concentration of 5 or 10% without inhibiting the PCR efficiency. However, the melting temperature of the primer will be reduced (21). Alternatively, to minimise the risk of secondary structures, either increase the annealing temperature in the PCR conditions or perform a nested PCR procedure.

Direct sequencing of PCR products by T7 DNA polymerase is prone to problems in GC-rich regions, due to the stability of secondary structure. To overcome these difficulties, Zhang *et al.* (184) have examined the effect of adding formamide to the sequencing reaction. Inclusion of formamide may increase the intensity of specific bands, dissolve several local secondary structures, and eliminate the presence of most bands which cross over all four lanes.

Sequencing primers
For genomic sequencing, internal sequencing primers are recommended. Preferably use different primers in the sequencing reaction as used for generating the PCR product. This is to reduce the risk of producing sub-optimal sequence data due to PCR artefacts (i.e. "false" run offs due to truncated PCR products) and high background because of false PCR products. However, under optimal conditions using nested PCR, the non-biotinylated primer used in the second PCR reaction may also be used as the sequencing primer (24).

Sequencing of GC-rich regions
The solid-phase approach is also the method of choice for sequencing of regions with high GC content (i.e. promoter sequences). Template preparation with Dynabeads Streptavidin permits sequencing of DNA containing GC-rich regions (typically 70-75%) with T7 DNA polymerase without the need for special conditions (except for the use of 7-deaza-dGTP instead of dGTP in the sequencing mixture (70)). This has been conducted in the fluorescence sequencing of a cloned *Streptomyces curacoi* gene.

Sequence analysis of a polymorphic region of the human dopamine D4 receptor gene has identified 19 unique, highly related 48 base pair repeat sequences using Dynabeads Streptavidin for the template preparation (90). This polymorphic region has GC contents in the range from 75 up to 87%. In this case, 10% DMSO was used in the reaction mixtures for both the PCR amplification and the sequencing reaction. For one of the two sequencing kits used, the dGTP was replaced by 7-deaza-dGTP.

Standard dideoxy sequencing using double-stranded DNA templates of this region was not feasible as the high GC content leads to unreliable sequence data (90).

Avoiding mispriming of DNA template

Mispriming of DNA template is one of the most common problems associated with the dideoxy chain termination method of DNA sequencing (143). It can be a particular problem with GC-rich primers and templates, or with templates containing repeats and unknown sequences. Thomas *et al.* (250) have found that a simple stringency wash can eliminate mispriming completely when using Dynabeads Streptavidin for the generation of single-stranded DNA templates. The Dynabeads/template/sequencing primer complex was washed twice with pre-warmed Sequenase reaction buffer, using the magnet stand (Dynal MPC) in the water bath, to separate the Dynabeads between the washes.

Figure 9 shows the results of sequencing part of the human mitochondrial DNA D-loop region with and without a stringent wash of the complex. The stringency wash proved effective both in removing cross-banding due to mis-priming and in increasing the signal generated from correctly primed template DNA. Furthermore, this improvement occurred over a broad temperature range (45 to 55°C) and with a minimum number of washes (two). This method can only be performed on an immobilised single-stranded DNA template which can be separated from the washes.

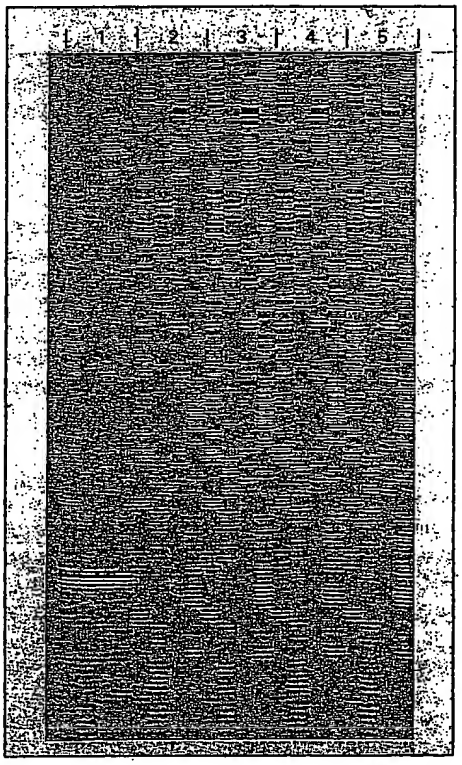


Figure 9. Autoradiagram of sequencing gel showing five sets of sequencing reactions, each containing identical template DNA and sequencing primer combinations, but different in their treatment after sequencing primer annealing (250). Sequencing reactions were carried out on sample 1 with no further processing. Sample 2 and 3 were washed once and twice respectively at 45°C and sample 4 and 5 washed once and twice respectively at 55°C. An internal sequencing primer was used.

Preparative isolation from agarose gel

Two separate research groups have described preparative isolation from agarose gel (16, 53). In one (53), the PCR reaction product was separated

in 1% low melting agarose gel. The relevant band was cut out and melted at 80°C for 5 min on a heating block with Dynabeads Streptavidin. The high temperature melted the gel and denatured the DNA. The biotinylated DNA strand bound to the Dynabeads Streptavidin to obtain single-stranded templates for sequencing, whilst the non-biotinylated strand was removed by incubation with 0.15 M NaOH for 5 minutes at room temperature. In the other group (16) a similar approach was used. The PCR product was separated in a 0.8% NuSieve® agarose gel. The relevant band was cut out and melted at 65°C for 10-15 min on a heating block. An equal volume of 2 x B&W buffer was added, together with Dynabeads Streptavidin. The suspension was incubated at 43°C for 30 minutes to immobilise the template onto the Dynabeads. Following two wash steps in B&W buffer and two wash steps in TE-buffer, the non-biotinylated DNA strand was removed by incubation in 0.1 M NaOH for 10 minutes at room temperature.

In both protocols, the immobilised template was further washed in NaOH and then distilled water before resuspension in the appropriate buffer for DNA sequencing.

Dynabeads Streptavidin used directly in PCR
The unique formulation of Dynabeads Streptavidin enables them to be used directly in PCR. The presence of Dynabeads in the reaction will not affect the amount of PCR product if the following guidelines are observed (39, 103, 125):

Table 4: Dynabeads in PCR-reactions. All PCR reactions were set up in the same way: 10 x PCR buffer (500 mM KCl, 100 mM Tris pH 8.3, 15 mM $MgCl_2$, 0.01% gelatin, 2 mM dNTP, 1 U AmpliTaq, 5 pmol primers). PCR programme (25 cycles, 95°C 15 sec; 65°C 1 sec; 72°C 1 min) Perkin Elmer 9600 cycler. 1 x B&W buffer (2 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA). Signal is measured as visible PCR product on a 0.8 - 1.5% agarose gel.

µg particles (per 50 µl total PCR reaction volume)	Dynabeads Streptavidin	Competitor 1 Streptavidin	Competitor 2 Streptavidin
250	-	-	-
150	+	-	-
100	++	-	-
50	+++	-	-
20	+++	-	-
10	+++	+	++
5	+++	++	+++
2	+++	+++	+++
1	+++	+++	+++
0.5	+++	+++	+++
0.2	+++	+++	+++
0	+++	+++	+++

+++: good signal ++: fair signal +: weak signal -: no signal

1. Wash Dynabeads coupled to DNA template once with PCR buffer using magnet stand (Dynal MPC).
2. Resuspend Dynabeads in PCR buffer with nucleotides, primers and *Taq* polymerase.
   Note: A maximum of 50 µg Dynabeads Streptavidin are used in 50 µl PCR reaction volume. This is s 10 times greater limit compared to other magnetic particle competitors (see Table 4).

3. Perform the full PCR cycling reaction with the Dynabeads present.
   Note: If desired, the Dynabeads may be removed after the first cycle by magnetic separation. The supernatant should be pipetted to another PCR tube to complete the cycling reaction. The Dynabeads may be stored in TE buffer at 2-8°C for later use.
4. Analyse the amplified PCR products by agarose gel electrophoresis.

2.6 Reference applications

2.6.1 Clinical DNA sequencing

Unpredictable mutations in somatic cells are responsible for the majority of genetic diseases. Genes involved in cell proliferation are often targets for acquired mutations, which have been found to occur in regions of the DNA sequence that disturb either protein function or transcriptional and/or translational control.

Direct sequencing of PCR-amplified material is the technology of choice for the emerging field of clinical DNA sequencing in the investigation and research of genetic diseases e.g. basal cell carcinoma (63), Bowen's disease (18), familial defective apolipoprotein B (FDB) (88). Using the Dynabeads system for direct sequencing, all demonstrate that single point mutations may be easily detected. The nucleotide sequence of infecting bacteria or viruses can also be determined directly from patient samples.

Sequencing with Dynabeads precisely defines the location and nature of mutational changes and represents the ideal mutation scanning technique. An example of direct clinical DNA sequencing is shown in Figure 10. This figure illustrates a point mutation appearing in the p53 gene encoding a nuclear phosphoprotein which is amongst the most frequent genetic alteration in human solid tumours. The sequencing data easily detects a C→T transition in tumour DNA in a skin cancer study (18).

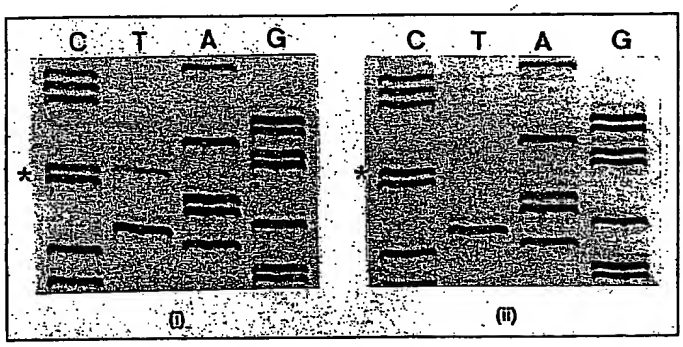


Figure 10. (i) Tumour DNA, showing C→T transition at codon 248 (*). (ii) Normal sequence at codon 248 (*) (18).

2.6.2 Sequence-based HLA typing

The HLA system plays a central role in controlling immune responses and influencing susceptibility to a large number of diseases. Both hybridisation with sequence-specific oligonucleotides (PCR-SSO) and amplification with sequence-specific primers (PCR-SSP) have already found great favour in the HLA-typing area (13).

Sequence-based typing (PCR-SBT) is playing an increasingly important role in research on tissue typing for transplantation (162). Direct solid-phase DNA sequencing in particular, with its reliability, its capacity for

revealing the entire nucleotide sequence coding for any HLA antigen, and its ability to detect variations as slight as a single base change, offers the potential of providing a comprehensive solution to tissue-typing (162). Furthermore, this technique is amenable to automation and is potentially ideal for routine clinical use (162).

Kaneoka *et al.* (75, 76) have established a method for typing HLA-DR genes by direct sequencing of PCR amplified DNA using Dynabeads Streptavidin as the solid support. Spurkland *et al.* (152) have also established a similar method for HLA-DR and HLA-DQ genes. Sequencing ladders obtained by these procedures are easily readable. The patterns can be interpreted in HLA homozygous, as well as heterozygous, individuals. Sequence differences or similarities between the bone marrow transplantation donor and recipient can be directly identified (152).

PCR-SBT of HLA-DPB alleles using Dynabeads Streptavidin for the template preparation has been compared to PCR-SSO and results were found to correlate perfectly (171).

In certain clinical sequence-based analysis (i.e. mutation screening), PCR amplification and direct DNA sequencing of a specific HLA locus with high degree of polymorphism (i.e. HLA-DQB1) in parallel, allows the creation of a genetic "fingerprint" for each patient (63). This can be used to ensure samples do not become muddled.

Both manual and automated sequencing techniques using a variety of sequencing strategies have been used for sequence-based typing. A summary of the different HLA loci, which have been determined by sequence-based typing using Dynabeads as the solid support, is presented in Table 5.

Table 5: Sequence-based typing of different HLA loci using Dynabeads as the solid support and choice of sequencing strategy.
ABI373A is the sequencer from Perkin Elmer - Applied Biosystems Division, Foster City, CA, USA.
ALF is the sequencer from Pharmacia Biotech AB, Uppsala, Sweden.
DNA4000 is the infrared sequencer from LI-COR, Inc., Lincoln, NE, USA.

HLA locus	Sequencing strategy	Reference
HLA-DPB1	Automated sequencing (*Taq* Dye Primers) - ABI373A	162
HLA-DPB1	Automated sequencing (*Taq* Dye Primers) - ABI373A	171
HLA-DPB1	Automated sequencing (T7 Dye Primers) - DNA4000	80
HLA-DQB1	Manual sequencing (^{35}S)	152
HLA-DQB1	Automated sequencing (T7 Dye Primers) - ALF	63
HLA-DQB1	Automated sequencing (T7 Dye Primers) - ALF	64
HLA-DQB1	Manual sequencing (^{35}S)	80
HLA-DQB1	Automated sequencing (T7 Dye Primers) - DNA4000	80
HLA-DRB1	Manual sequencing (^{32}P)	75
HLA-DRB1	Automated sequencing (*Taq* Dye Primers) - ABI373A	76
HLA-DRB1	Manual sequencing (^{35}S)	152
HLA-DRB1	Automated sequencing (T7 Dye Primers) - DNA4000	80
HLA-DRB3	Automated sequencing (T7 Dye Primers) - DNA4000	80
HLA-DRB4	Automated sequencing (T7 Dye Primers) - DNA4000	80
HLA-DRB5	Automated sequencing (*Taq* Dye Primers) - ABI373A	76
HLA-DRB5	Automated sequencing (T7 Dye Primers) - DNA4000	80
RING4 gene	Manual sequencing (^{32}P)	78
RING11 gene	Manual sequencing (^{32}P)	78
RING11 gene	Manual sequencing (^{32}P)	119

2.6.3 Sequence-based bacterial typing

Sequence analysis of ribosomal RNA (rRNA) genes is useful for identification of, and discrimination between, closely related bacterial species (74, 108). In general, ribosomal DNA (rDNA) complementary to the rRNA is generated by PCR. rRNA sequences can be used for species identification using a rRNA reference sequence library, and generic PCR primers from conserved areas of prokaryote rRNA genes can be used for most bacterial species (108). Data corresponding to 400-600 bases from 10-20 strains can be obtained within a single working day (109).

DNA sequencing has also been demonstrated to be a useful epidemiological tool for identification and characterisation of clinically important isolates (108). Such information can be used for investigation of sources and routes of transmission.

Sequence analysis of rRNA genes have also applications in evolutionary studies and for diseases associated with unculturable bacterial strains (127). The 16S rRNA sequence mutates at a rate that makes it useful as an 'evolutionary clock'; the evolutionary distance from one organism to another can be calculated from the number of nucleotide differences between their respective 16S rRNA sequences. Because portions of all 16S rRNA genes are highly conserved, these genes can be amplified from uncharacterised organisms with broad-range primers used in the PCR. This method has been used to identify a unique Bacillus species, the unculturable organism responsible for Whipple's disease (127).

Automated solid-phase DNA sequencing with T7 DNA polymerase can be used for efficient generation of sequence data from rRNA genes (116). Primary data with more even peak heights are obtained by sequencing with T7 DNA polymerase than by other sequencing methods. The results indicate that direct solid-phase DNA sequencing procedure using Dynabeads Streptavidin is a powerful tool for identification of mycoplasmas and is also useful in taxonomic studies (Pettersson *et al.* 1994). Furthermore, the method is suitable for diagnostic applications in bacteriology, and will be particularly useful when more rRNA sequences have been added to the database (116). Bacterial typing based on direct automated solid-phase DNA sequencing using Dynabeads has the following advantages (115):

- Reverse transcription is not needed. (This depends on whether rRNA or rDNA is used as starting material; reverse transcription of rRNA may be preferred as starting material, because of the high copy number per cell.)
- Cloning procedures are not required.
- Analysis of non-cultivable species is possible.
- The Dynabeads solid-phase procedure is suitable for automation.

A universal and reliable sequencing approach suitable for complete automation for evolutionary and phylogenetic studies of most plant species, based on chloroplast DNA analysis, has also been developed (41). In a similar manner, sets of nested or hemi-nested primers have been made to amplify the highly conserved chloroplast trnL and trnF genes. PCR products obtained from these regions in the chloroplast genome from a variety of species have been sequenced. The results have shown that these regions are useful in phylogenetic studies at different taxonomic levels (41).

Sequence-based bacterial typing using Dynabeads as solid support is summarised in Table 6.

Table 6: Sequence-based bacterial typing of different bacterial species using Dynabeads as the solid support with belonging target rRNA gene.

	Target gene/sequence	Reference
Campylobacter	23S rRNA gene	40
Campylobacter jejuni	23S rRNA gene	167
Escherichia coli	16S rRNA gene	116
Legionella bozemanae	5S rRNA gene	131
Legionella pneumophila	5S rRNA gene	131
Magnetotactic bacteria	ssu rRNA gene	30
Mycoplasmas	16S rRNA gene	115, 116

2.6.4 Forensic and population studies

Sequencing of mitochondrial DNA
Sequence analysis of mitochondrial DNA (mtDNA) has applications in forensics, population and human diversity studies, genealogical studies, and species identification.
Mitochondrial DNA exists in mitochondria of all animal cell types as a closed, circular, double-stranded molecule. Human mtDNA is 16,569 bp in length (113). There are several characteristics of mtDNA that make it a useful tool for human identification. The following examples illustrate instances in which mtDNA analysis may be preferred over nuclear DNA.

1. **A sample containing limited amounts of DNA, such as a hair shaft.** Because mtDNA is present in high copy number (single mammalian cells contain from several hundred to 10,000 mitochondrial genomes) the chances of successful PCR amplification even from very small numbers of cells are good.
2. **Samples that are highly degraded, such as ancient remains (i.e. bone and teeth).** Because mtDNA is circular, it is less susceptible to exonuclease degradation. Because of the high copy number, the chances that an un-degraded fragment will survive is greater.
3. **Samples of remains where only distant relatives are known.** Because mtDNA is maternally inherited, as long as an individual shares maternal descent with a candidate sample source, he or she can be used to verify identity (51).

In order to develop a robust protocol for the automated analysis of mtDNA from semen and hair shaft, four different amplification and sequencing strategies have been compared (67). Optimal results were obtained using Dynabeads followed by solid-phase DNA sequencing with Sequenase. This sequencing strategy appeared to be the most reliable, the most sensitive and the most consistent of the methods.

Sullivan *et al.* (154) have continued the development towards a fully automated forensic DNA test where the solid-phase approach has been used for the analysis of amplified hypervariable segments of mtDNA. This approach enabled good quality sequences to be generated from severely degraded and low-concentration DNA samples, such as from human bones buried for over 50 years, thereby providing a definitive identification test. This method have also been used to sequence mtDNA from 100 unrelated British Caucasians to investigate the nucleotide diversity (118).

- Nine skeletons found in a shallow grave in Ekaterinburg, Russia, in 1991, were tentatively identified by Russian forensic authorities as the remains of the last Tsar, Tsarina, three of their five children, the Royal Physician

and three servants. Sequence analysis of mtDNA using Dynabeads Streptavidin revealed an exact sequence match between the putative Tsarina and the three children with a living maternal relative (51). The authors concluded that the DNA evidence supported the hypothesis that the remains are those of the Romanov family.

Solid-phase sequencing has also been used in a population study to examine nucleotide sequence variation in the mtDNA from 90 humpback whales collected from the three major oceanic basins (10).

Forensic evidence by direct genomic sequencing
Probably the first time that evidence produced by DNA sequencing was used in court was in a rape case when direct genomic sequencing was used to compare HIV-1 pol and gag genes sequences from the male defendant and the female victim (4, 5). The direct sequence analysis using solid-phase DNA sequencing showed that the virus populations harboured by the male and the female were highly homologous. This case demonstrated the power of direct genomic sequencing in forensic medicine (5).

2.6.5 Serial analysis of gene expression

Gene expression differences between cells and tissues can be compared by techniques based upon cDNA subtraction or differential display, but such methods provide only qualitative data. Expressed sequence tags (ESTs) are invaluable for investigating differential gene expression, but large-scale EST sequencing is very expensive. Serial analysis of gene expression (SAGE) is a novel method for analysing levels of gene expression. The original work (170) describes a rapid screening of gene messages in cells or tissues, allowing the quantitative and simultaneous analysis of thousands of transcripts. Tags can be analysed for abundance and identity and quantitation of expression. SAGE can indicate quantitative and qualitative alterations in gene expression in normal samples, diseased states and during development.

SAGE is based upon two principles. First, short nucleotide sequence tags (as small as 9 base pairs) contain sufficient information to identify a transcript isolated from a defined position. Secondly, concatenation of the tags allows analysis of transcripts in a serial manner on a sequencing gel. Dynabeads Streptavidin provide the most simple and efficient way to capture the signature tags.

Outline protocol for SAGE

For further details on this protocol it may be beneficial to contact the original authors, Velculescu *et al.* (170).

1. Isolate mRNA from the tissue or cells and transcribe to cDNA with biotinylated oligo (dT) primer.
2. Cleave the double-stranded cDNA with frequent cutting restriction enzymes that cleave the DNA more than once.
3. Capture the 3' ends onto Dynabeads Streptavidin.
4. Divide and ligate the cDNA via the restriction site to one of 2 linkers that contains a type IIS restriction site.
5. Generate the signature tags, containing linker sequences and a short piece of cDNA using type IIS restriction endonucleases which cleave at a site 20 base pairs away from their recognition site.
6. Ligate released tags together and use as a template for PCR, thus creating a ditag. Use primers specific for the linker region.

7. Cleave PCR product to isolate ditags.
8. Concatenate, clone and sequence multiple ditags.

The extensive handling steps of the SAGE procedure results in the Dynabeads being in plain buffer for long periods. Addition of one or more washing steps, with 0.02% Tween-20 or a small concentration of SDS added to a Washing or Binding Buffer is helpful. Addition of BSA may also assist. However, care must be taken that these additions will not affect subsequent enzymatic modification steps.

3. Protein interactions and gene regulation

3.1 Purification of sequence-specific DNA/RNA binding proteins

DNA and RNA binding proteins (e.g. promoters, gene regulatory proteins and transcription factors) are of major importance both for normal cellular control and in disease states such as cancer. Unfortunately, these molecules are often short-lived and in low abundance.

In order to study these molecules successfully, they must be highly purified using a rapid and sensitive method. A DNA affinity purification method first developed by Gabrielsen *et al.* (46, 47) describes the use of Dynabeads Streptavidin for this application. The technique is outlined in Figure 11.

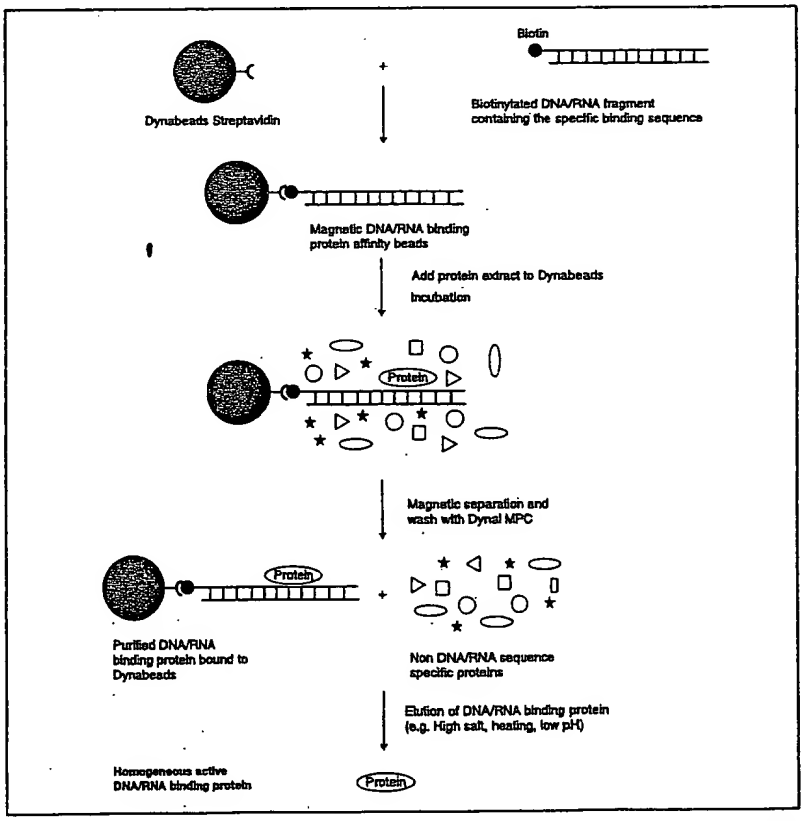


Figure 11. Diagram of method for purification of strand-specific DNA/RNA binding proteins using Dynabeads Streptavidin.

The Dynabeads Streptavidin solid-phase method is based on the need for a biotinylated DNA/RNA sequence motif to which the protein of interest will specifically bind. If this motif is bound to Dynabeads Streptavidin, then a reusable, magnetic solid-phase with affinity for the target protein is created. The magnetic DNA/RNA affinity beads can then be mixed with the nuclear extract and binding is completed within minutes. Rapid purification allows for the simple isolation of unstable gene regulatory proteins.

A wide range of sequence-specific DNA/RNA binding proteins have been purified using this technique (please refer to Table 7 below). Particular interest has been generated by the following results:

- Yeast transcription factor tau (τ) has been purified to near homogeneity in less than 30 minutes and is fully active in transcription and DNA binding assays (46, 47).
- A DNA binding protein, MS2, from C_2 murine skeletal myotubes has been purified (128).
- The ecdysteroid receptor (EcdR) from *Drosophila melanogaster* has been enriched 29,000 fold within 90 minutes (110).

Table 7: Sequence specific DNA/RNA binding proteins purified using Dynabeads Streptavidin to create a magnetic DNA/RNA affinity bead with associated target binding sequences and elution conditions.

Binding protein	Target binding sequence	Elution condition	Reference
Cellular transcription factor RBP-Jκ	Double-stranded DNA	High salt buffer	286
Cellular transcription factor RBP-Jκ	Double-stranded DNA	High salt buffer	83
cMyb protein	Double-stranded DNA	No elution	48
DNA topoisomerase I	The 5'-phosphate end of a cleaved DNA strand	High concentration of nucleotides in a high salt buffer	10
Drospophila heat shock factors and GAGA factors	Plasmids	No elution (DNase I footprinting)	140
Ecdysteroid factor	Double-stranded DNA	High salt buffer	110
FMR1 protein	Single-stranded RNA	Released by heating	9
Hepatic protein, p27	Guanine rich single-stranded DNA	High salt buffer	124
Human replication protein, RPA	DNA	No elution	61
Initiation protein of DNA replication in E.coli, DNAa	mAb-β-galactosidase	98% formamide	132
Initiator protein RepC	Single-stranded DNA	Alkali treatment	126
LacI-β-gal fusion protein	Double-stranded DNA	DNase I treatment	91
Preprotachykinin	Single-stranded DNA	High salt buffer	121
Protein factor MS2	Double-stranded DNA	Low pH	128
Single-stranded telomere binding protein (sTBP)	Single-stranded $TTAGGG_n$ repeats	High salt buffer	95

Transcription factor, γRF-1 induced by IFN-γ, human monocytes and epidermal cells	DNA containing γRE-1 sequences	0.6 M KCl	42
Transcription factor, nuclear factor I (NFI) in rabbit reticulocyte	DNA	No elution (depletion)	36
Transcription proteins, STAT, in human myeloid cells	G-SIF-A, HSIE	High salt buffer with NaCl	20
Vaccinia virus early transcription factor (VETR)	Double-stranded DNA	High salt buffer	49
Xenopus tissue specific in vivo DNA binding proteins, Spa, AP2	DNA	No elution (DNase I footprinting)	174
Yeast transcription factor tau (τ)	Double-stranded DNA	High salt buffer	46, 47

3.1.1 Advantages of using Dynabeads Streptavidin for purification of DNA/RNA binding proteins

Dynabeads Streptavidin provide a superior alternative to affinity chromatography for the purification of low abundance, unstable, sequence specific DNA or RNA binding proteins. The major advantages are listed below.

- High yields of enriched proteins are obtained using only one adsorption step (47).
- The method is convenient and rapid. Washing and elution steps benefit from Dynabeads 'magnetic handling' property, making isolation of proteins possible in one hour. In the case of purification of yeast transcription factor tau (τ), higher purity was obtained in less than one hour using Dynabeads than was normally obtained with the same starting material after three days and three columns (46).
- The high stability and binding capacity of the DNA on the Dynabeads allows binding of the target proteins with kinetics similar to that of DNA in free solution (47).
- DNA coupled to Dynabeads Streptavidin is reusable at least ten times (128).
- Improved purity and yields are obtained by saturating the DNA affinity beads with the specific DNA binding protein and by including competitor DNA only during washing of the beads (47).

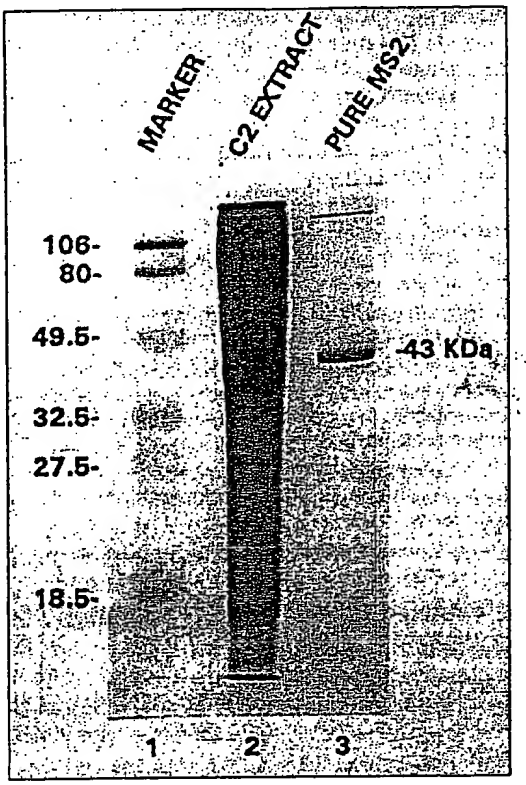


Figure 12. Comparison of C_2 murine skeletal myotube extract and purified MS2, a DNA binding protein (128). Denaturing gel, 12% SDS-PAGE; stained and photographed. Lane 1: 5 µl prestained marker proteins; lane 2: 100 µg C2 cell extract; lane 3: 0.5 µg MS2 magnetically purified from C2 cell extract. MS2 seen to be a single protein of 43 kDa.

3.1.2 Materials required

- Dynabeads M-280 Streptavidin (10 mg/ml) (Product No. 112.05/06)
- Magnet stand, DynaMag-2 (see Chapter 1, section 3, page 15)
- Biotinylated DNA/RNA sequence containing the actual binding domain for your target protein (see Chapter 5, section 5, page 165 for biotinylation procedures)
- Binding & Washing buffer for immobilising the biotinylated DNA/RNA sequence to Dynabeads Streptavidin (see section 2.3, page 101 for recipe)
- Crude or partly purified protein extract
- Competitor DNA (optional)
- Protein binding buffer
- Protein wash buffer
- Protein elution buffer
- Tilting/rotation apparatus (e.g. Dynal MX1)
- Sterile test tubes, glasswate pipettes

3.1.3 Protein binding, washing and elution buffers and conditions

Gabrielsen *et al.* (47) provide some helpful hints for optimising protein binding, washing and elution conditions. In addition, several other publications describe purification conditions used for particular extractions.

1. Yeast transcription factor tau (τ) (47)

Binding: Protein binding was performed in a TGED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.01% Triton® X-100) with 100 mM NaCl and mixed at 25°C for 5 minutes.

Washing: Dynabeads were washed once with a TGED buffer containing 100 mM NaCl, once with TGED buffer containing a 10-fold excess of poly[d(I-C)] competitor DNA, and once in TGED buffer containing 100 mM NaCl.
Elution: The bound protein was eluted with 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.01% Triton X-100 and 1 M NaCl on ice.

2. Protein factor MS2 (128)
Binding: Protein binding was performed in 5x gel shift buffer (1x gel shift buffer: 20 mM Tris-HCl, pH 7.0, 100 mM NaCl, 5 mM EDTA, 0.1% Nonidet® P-40 [NP-40], 0.5 µg/µl BSA and 5% glycerol), 1.25 µl 1 M $MgCl_2$, 0.378 ml dialysis buffer, and mixed at room temperature for 30 minutes.
Washing: Dynabeads were washed three times with 1x gel shift buffer containing 2.5 mM $MgCl_2$ and 0.1% NP-40, but lacking BSA.
Elution: The bound protein was eluted with 100 mM sodium acetate, pH 4.2.

3. Single-stranded telomere binding protein (sTBP) (95)
Binding: Protein binding was performed with 20 µg sonicated *E. coli* DNA, 15 µg oligonucleotide B575 in 1 ml 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4% glycerol, 0.1% Triton X-100, 10 mM β-mercaptoethanol, and mixed at room temperature for 20 minutes.
Washing: Unspecified.
Elution: The bound protein was eluted with two 100 µl washes of 20 mM Tris, 1 mM EDTA, 15% glycerol, 0.05% NP-40 and 1 M NaCl.

4. *Vaccinia* virus early transcription factor (VETR) (49)
Binding: Protein binding was performed with buffer A (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.01% NP-40, 1 mM dithiothreitol, 10% (v/v) glycerol) containing 70 mM NaCl for 30 minutes.
Washing: Dynabeads were washed twice for 10 minutes each time with buffer A containing 100 mM KCl.
Elution: The bound protein was incubated three times with buffer A containing 400 mM KCl and once with buffer A containing 2 M KCl.

5. Ecdysteroid receptor (EcdR) (110)
Binding: Protein binding was performed in 20 mM Hepes/KOH, 110 mM KCl, 2 mM dithiothreitol, 1 mM EDTA, 2 mM KH_2PO_4, 10% (v/v) glycerol, pH 7.6, and mixed at 0-4°C for 30 minutes.
Washing: Dynabeads were washed four times with 20 mM Hepes/KOH, 110 mM KCl, 2 mM dithiothreitol, 1 mM EDTA, 2 mM KH_2PO_4, 10% (v/v) glycerol, pH 7.6.
Elution: The bound protein was eluted with 20 mM Hepes/KOH, 10% (v/v) glycerol, pH 7.6 and 400 mM KCl.

3.1.4 Binding capacity and storage

One 40-mer oligonucleotide primer linked to the bead surface containing one binding site for the target protein per oligo will bind approximately 2.5 - 5.0 µg of a 60 kDa protein per mg of Dynabeads Streptavidin.

To increase the yield, repeating specific recognition sequences can be made. Depending on the size of the binding protein, allow at least 10 ran-

domly synthesised nucleotides between the bead surface and the start of the recognition site.

The prepared magnetic DNA affinity beads can be stored at 2-8°C for several months without loss of DNA binding protein binding capacity. DNA affinity beads stored at 2-8°C for more than two years have been used successfully (47).

Please refer to Chapter 1, section 2.3.2 page 14 and Chapter 5, section 2.3, page 158 for binding capacity and optimal binding conditions for coupling of biotinylated DNA/RNA to Dynabeads Streptavidin.

When working with RNA binding proteins, it is important to prepare the Dynabeads Streptavidin for RNA work (see Chapter 5, section 1.2 page 156) and to treat all solutions, plasticware and other current equipment with diethyl pyrocarbonate (DEPC) to avoid degradation of RNA by RNases.

Caution: DEPC is a suspected carcinogen and should be handled with care.

3.1.5 Protein purification protocol

There are a number of factors to consider when developing a protocol, including the degree of partial purification required for the specific protein to be isolated. This will depend upon the following criteria:

- The protein
- Its source
- Its abundance
- Its binding affinity
- The amount of other contaminating DNA/RNA binding proteins
- The presence of nucleases
- The degree of partial purification
- pH and temperature dependence of binding/elution

Additionally, consideration should be given to salt molarities for binding, wash and elution, the effect of added competitor DNA and the saturation conditions of protein to DNA/RNA. Due to Dynabeads flexibility and unique 'magnetic handling' which enables rapid buffer changes, optimisation of reaction conditions should be readily achieved. A detailed review of the relevant considerations has been compiled (47).

Detailed examples of binding, wash and elution conditions are described in section 3.1.3 above. Direct and indirect capture is possible (see Figure 11). The following is a general outline of the protein purification procedure.

1. Prepare the protein extract.
2. Add Dynabeads Streptavidin coated with appropriate DNA/RNA sequence to protein extract.
3. Incubate Dynabeads with protein extract whilst providing bi-directional mixing (e.g. Dynal mixer).
4. Place tube containing the Dynabeads with bound protein extract in a magnet stand (Dynal MPC).
5. Remove supernatant and wash Dynabeads several times with buffer using magnet stand.
6. Elute protein by resuspending Dynabeads and bound protein in an elution buffer.

7. Separate Dynabeads and eluted protein using magnet stand.
8. Proceed with analysis of the purified protein.

3.1.6 Regeneration of the DNA/RNA affinity Dynabeads for reuse

Reuse of Dynabeads Streptavidin for purification of DNA binding proteins has been described (47, 128). The following quotes may assist in deciding upon regeneration procedures.

Gabrielsen *et al.* (47): "Used beads are regenerated by repeated washes in high salt buffer (TGED with 2 M NaCl or TGED with 2 M NaCl and 6 M urea) and washed in TEN (TE buffer with 100 mM NaCl) buffer and are stored at 4°C for further use."

Ren *et al.* (128): "After elution of purified protein, the beads should be regenerated without delay, if beads are to be reused. DNA and beads were reusable at least ten times if completely washed after each use and stored at 4°C. Beads, with or without tether s4, were prepared for storage and reuse by washing twice with TE buffer, pH 8.0."

3.2 Determination of consensus DNA sequences for DNA binding proteins

Study of regulation of gene expression requires a technique for determining the DNA sequence that is recognised by a specific DNA binding protein. Since individual sequences of interest often represent a very small percentage of the total sequences present, separation and amplification of these sequences is necessary. Kinzler and Vogelstein (79) describe a method where a combination of affinity selection of the DNA, immunoprecipitation and PCR amplification of the enriched DNA is performed. This selection procedure is repeated and amplified recovered sequences can be cloned and/or used as hybridisation probes.

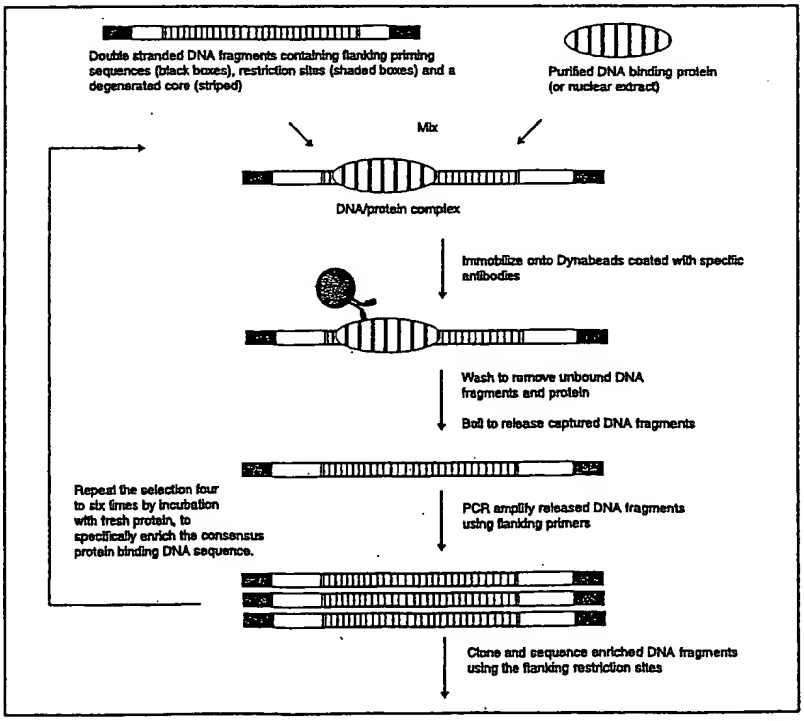


Figure 13. Schematic diagram illustrating how Dynabeads coated with monoclonal antibodies against DNA binding proteins can be used to investigate the consensus DNA sequence binding site.

Wright *et al.* (179, 180) describe a similar technique to determine the consensus/optimal binding site for a DNA binding protein, based upon the immunocapture of the DNA/protein complex using Dynabeads coated with monoclonal antibodies specific for the DNA binding protein being studied. In this technique, termed CASTing (Cyclic Amplification and Selection of Targets), an oligonucleotide consisting of a degenerate core flanked by restriction sites and with defined sequences that can serve as priming sequences at both ends, is synthesised as a first step. The oligonucleotide is made double-stranded by priming the DNA synthesis with the 3' end primer.

The resulting double stranded DNA consists of:
An upstream primer sequence - a restriction site region - the degenerated core - a second restriction site region - a downstream primer sequence.

This double-stranded DNA is mixed with purified DNA binding protein or with nuclear extract and the DNA-protein complex is immunoprecipitated with Dynabeads coated with monoclonal antibodies specific for the protein of interest.

Either Dynabeads coupled with specific monoclonal antibodies or biotinylated antibodies coupled to Dynabeads Streptavidin are suitable for this procedure.

The DNA recovered from the first cycle of selection is released by boiling the Dynabeads-protein-DNA complex and then amplified by PCR using the flanking primers. The amplified DNA is mixed with fresh protein and subjected to additional cycles of selection, including incubation with protein, immunoprecipitation and reamplification. At each cycle, the ratio of specific binding sites to random sequences is increased until sufficient specificity is obtained to justify cloning and sequencing the DNA. One cycle takes about 2.5 hours and typically four to six cycles are performed (47). The enriched DNA fragments can be readily cloned using the restriction sites introduced at each end of the degenerated core thus allowing analysis of individual sequences.

3.2.1 Technical tips

- Each cycle enriches for the highest-affinity sites and thus the ultimate selection is for the single highest-affinity interaction. Therefore, in order to observe a variety of multicomponent complexes, the number of cycles of selection must be limited to the minimum necessary to eliminate most of the non-specific sequences.
- If nuclear extracts are used as the protein source, a substantial fraction of the oligonucleotides will bind non-specifically. To reduce this non-specific binding a competitor (e.g. sonicated salmon sperm DNA (179, 180) or poly(dI-dC) (79) can be included.
- Over-amplification of the DNA should be avoided for two reasons. First, over-amplification of the DNA can result in production of very large artefactual DNA (179). Second, because the population of molecules being amplified during each cycle contain common 5' and 3' end (the PCR priming sequences) but differ in the central region, reannealing of these products will produce molecules with double-stranded ends and single-stranded middles (180).

3.3 Study of multi-component protein complexes bound to DNA/RNA

Dynabeads Streptavidin are not only useful in studies on binding properties of individual proteins and the nucleic acid sequences they recognise.

They have also been demonstrated to be of value in the isolation and study of multi-component complexes bound to DNA.

3.3.1 Solid-phase DNAse I footprinting

Both the detailed interaction of regulatory proteins with particular DNA sequences *in vivo* (footprinting) (122, 123, 139, 141, 165, 174) as well as the *in vivo* conformation of the DNA to which regulatory proteins are bound (177) has been investigated using Dynabeads Streptavidin.

Whilst footprinting is a valuable technique for studying gene regulation and the binding properties of sequence-specific DNA binding proteins, the purification of DNA fragments following enzymatic digestion is extremely time consuming. By using Dynabeads Streptavidin to capture selectively genomic fragments generated by DNase digestion of intact nuclei prior to electrophoresis the footprinting technique is significantly more rapid and results are improved.

Figure 14 outlines the technique. The fragments are first labelled with biotin using ligase and a biotinylated linker tag. The fragments are then amplified by PCR with *Vent* polymerase and captured onto Dynabeads Streptavidin (Linker Tag Selection - LTS). Thereafter, the fragments are made single-stranded and used as templates for primer extension with labelled primer. The products of the extension reaction are analysed on sequencing gels.

Weak bands on the sequencing gel correspond to exposed regions of DNA devoid of protein, whereas strong bands indicate the nucleotides to which protein is intimately attached. Compared to conventional footprinting techniques the signal strength of the footprints is greatly enhanced and background greatly reduced when Dynabeads are used to selectively enrich the genomic fragments (122, 139, 141).

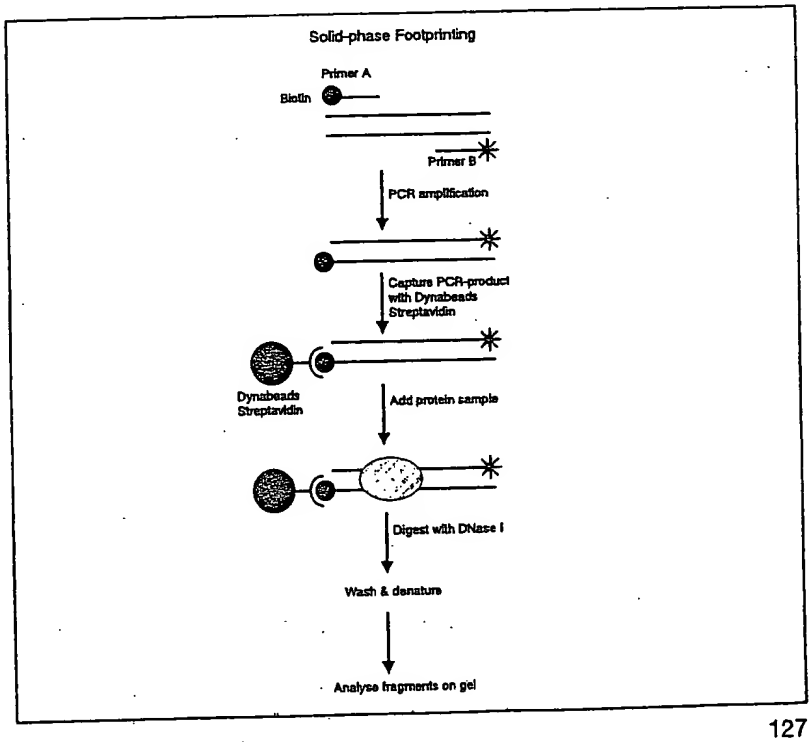


Figure 14. Outline of solid-phase footprinting. *For simple DNase I footprinting, a biotinylated primer (primer A) and a radiolabelled primer (primer B) are used in the PCR. Immediately following PCR amplification, the products are captured onto Dynabeads Streptavidin and protein sample added. The protein is allowed to bind to PCR products and all complexes are washed. The DNA is then digested with DNase I. Bound protein protects target DNA from digestion and absence of cleavage sites is revealed when the pattern of fragments is compared with DNA free of protein.*

By substituting fluorescein for the isotope on the second primer, fluorescein labelling of the DNA fragments can be used and the footprint patterns analysed using a standard automatic laser fluorescent DNA sequencer (136). This has several advantages over the traditional radioactive labelling, including elimination of radiation risks, improved stability for storage and absence of degradation by phosphatase.

Dynabeads Streptavidin have been used to isolate fragments of DNA to which biotinylated anti-Z-DNA antibodies had been cross-linked *in vivo*. Using this solid-phase footprinting technique, it could be demonstrated that the conformational state of a myc-promoter region changed from the B- to the Z-form depending on the state of transcriptional activation of the gene (177). Solid-phase footprinting has also been used to visualise the binding of *Drosophila* Heat shock Factor to the Heat Shock Element 1 of the hsp70 gene promoter (Figure 15) (136, 140).

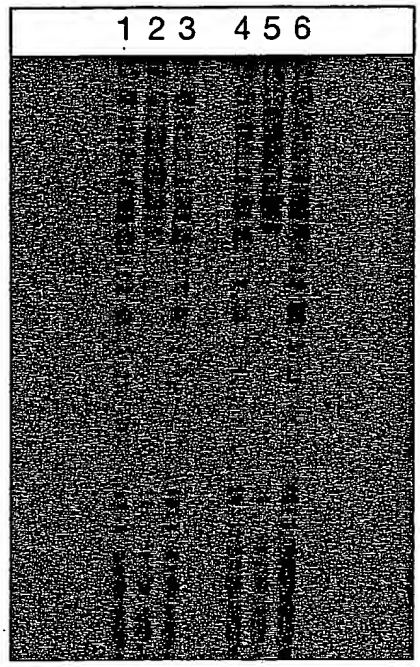


Figure 15. Solid-phase footprinting visualising binding of Drosophila *Heat Shock Factor (HSF) to Heat Shock Element 1 of the hsp70 gene promoter. Recombinant protein was allowed to bind to immobilised, radioactively labelled target fragment. The HSF footprint (lanes 2, 5) is deduced by comparison of fragment patterns derived from HSF/DNA complexes with the patterns of protein-free DNA (lanes 1,3,4,6). (137)*

Advantages

The major advantages of using Dynabeads Streptavidin in footprinting are listed below.

- Processing samples is rapid, with precipitations and centrifugations eliminated, and the time required reduced from 3 hours to 30 minutes.
- As only pure DNA fragments are loaded onto the gels, background is reduced and footprint results are of high quality and reproducible. (122, 139).
- Experimental design options are increased due to the strength of the streptavidin/biotin interaction and the ease of manipulating reaction conditions.
- Protein samples added to the DNA fragments can be crude. They do not require pre-purification as unspecific material is removed in wash steps.
- The unique 'magnetic handling' property of Dynabeads Streptavidin enables manipulation to be simple, rapid and efficient.

Protocol
The protocol provided below is based upon published methodology. As conditions must be optimised for each system, the protocol should be regarded as a guideline only.

PCR amplification
Use standard PCR with 50 pmol of each primer; one primer biotinylated at the 5' end and the second radio-labelled. Add 1 ng of template.

Product capture
1. 100 µl (1 mg) Dynabeads Streptavidin will bind up to 30 pmol of 1-4 kb double-stranded DNA. Use 300 µl (3 mg) Dynabeads Streptavidin. This will be sufficient for 20 reactions (if less reactions are required, scale down PCR and Dynabeads quantity accordingly).
2. Wash in Binding and Washing buffer (B&W buffer: 10 mM Tris-HCl, pH7.5; 1 mM EDTA; 2 M NaCl) using a magnet stand (Dynal MPC).
3. Resuspend Dynabeads/PCR product complex in B&W buffer to give final volume of 500 µl.
4. Mix for 15 - 30 minutes at room temperature.
5. Wash Dynabeads/DNA complex in B&W buffer using magnet stand.
6. Resuspend in 200 µl protein binding buffer.

Footprinting
1. For each reaction use approximately 15,000 cpm Dynabeads/DNA complex (10 µl resuspended volume).
2. Remove supernatant and add 25 µl protein binding buffer (choice of buffer is dependent upon protein) and 2 µg protein sample.
3. Incubate for 10 minutes.
4. Add DNase I. Amount of DNase I needed to produce 1 nick per DNA fragment must be determined (approximately 0.004 - 0.006 units).
5. Incubate for 1 minute.
6. Terminate reaction by adding an equal volume of 4 mM NaCl, 100 mM EDTA.
7. Wash once in 2 M NaCl, 20 mM EDTA and then once in 10 mM Tris-HCl pH 8.0, 1 mM EDTA to remove unbound protein.
8. Resuspend Dynabeads complex in formamide loading buffer.
9. Heat for 5 minutes at 76°C to denature.
10. Load and run on a denaturing polyacrylamide gel.
    Note: Removal of Dynabeads before loading is unnecessary.

Control
For control samples perform DNase I digestion on Dynabeads/DNA complex in the absence of added protein.

3.3.2 Complex assembly studies
Transcription initiation in eukaryotes by RNA polymerase II is preceded by the stepwise formation of a preinitiation complex (PIC). PIC formation rate can govern the rate at which a gene is transcribed. Transcriptional activator proteins (activators) can increase PIC formation rate. Using Dynabeads Streptavidin to isolate complexes formed *in vitro* under different conditions, it has been possible to show that the yeast activator GAL4 both increases the recruitment of the general transcription factor TFIIB into PICs and accelerates a step later in assembly (22).

3.3.3 Reconstitution of chromatin on Dynabeads
The reconstitution of chromatin on long (8 kb) DNA molecules attached to the Dynabeads has been used (138) to study transcription.

3.4 Selection from phage display libraries

Isolation of genes encoding proteins with known binding properties has relatively recently been facilitated by new separation technologies using phages or other vectors.

Phages are transfected with genes expressing the protein on the surface. The phage is then selected by binding affinity and amplified. The strong link between genes and encoded protein enables selection in rounds of binding and growth. Mammalian cells, filamentous bacteriophages and retrovirus have all been utilised for display of expressed proteins. Filamentous phages and retroviral vectors expressing functional antibody fragments or specific antibodies against an antigen have been used for improving and controlling the affinity of the antibody/antigen fragment, dissociation from the antigen and other parameters of antibody/antigen interaction (60, 133).

Dynabeads Streptavidin in combination with biotinylated haptens have been successfully used for the selection of vectors expressing proteins (see Figure 16). For example, after amplification in *E.coli*, M13/FD phage single-stranded DNA templates for subsequent sequencing can be isolated using Dynabeads Streptavidin combined with sequence-specific biotinylated oligonucleotide probes. Similarly, binding proteins have been selected from combinatorial libraries of an α-helical bacterial receptor domain using phage display, with phage capture using Dynabeads Streptavidin (107).

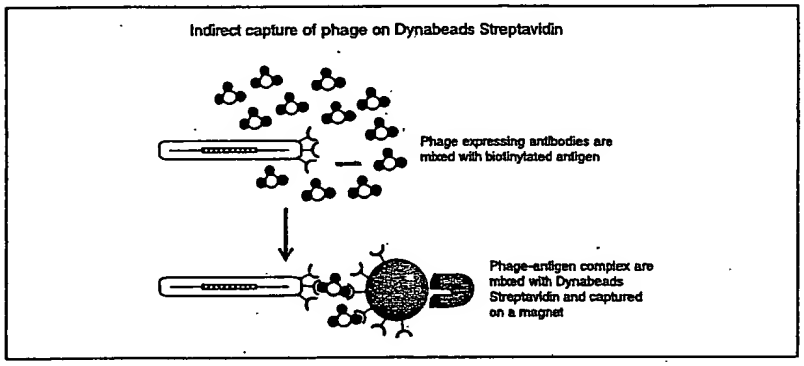


Figure 16. Schematic diagram showing the principle for selection of phages from a library using Dynabeads Streptavidin.

4. Immunomagnetic cell isolation

Biotinylated antibodies bound to Dynabeads Streptavidin provide a simple and rapid method for the positive isolation or negative depletion of cells. For example, cells can be isolated from whole blood, buffy coat or gradient isolated mononuclear cells.

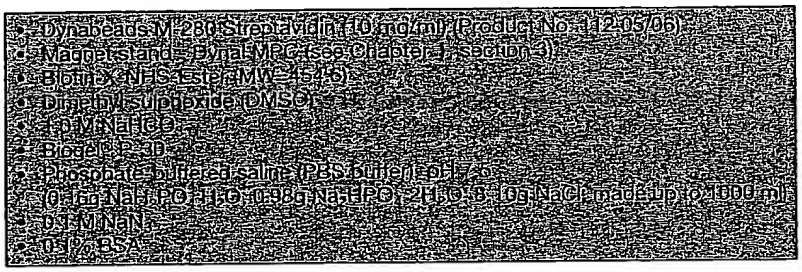
The widespread availability of commercially produced biotinylated antibodies makes Dynabeads Streptavidin particularly suitable for immunomagnetic cell separation. When performing cell depletions, a cocktail of biotinylated antibodies can be used in conjunction with Dynabeads Streptavidin.

Extremely fragile cell types may benefit from a slower rate of attraction speed of Dynabeads towards the magnet and for such cell types

Dynabeads coated with non-biotinylated antibodies may be more appropriate. For cell separation using non-biotinylated antibodies, Dynal offers Dynabeads coated with various secondary antibodies, as well as Dynabeads coated with specific antibodies against various cell antigens.

Tosylactivated Dynabeads are also available for direct coating with the users own antibodies.

4.1 Materials required (including buffers)



4.2 Protocols for immunomagnetic cell isolation

The large selection of commercially available biotinylated antibodies will frequently eliminate the need to biotinylate antibodies oneself. If the antibody required is unavailable in biotinylated form, the following protocol can be used for biotinylation (25, 28).

> The antibody to be biotinylated should either be a purified monoclonal or an affinity-purified polyclonal antibody. Sera are unsuitable for biotinylation.

1. Calculate number of purified antibody molecules per volume unit.
2. Dissolve 10 x molar excess of biotinylation reagent Biotin-X-NHS-Ester (MW=454.5) in 10 ml DMSO and add this solution to antibody solution. (For further information on recommended biotin, see Chapter 5, section 5, page 165).
3. Add required amount of a 1.0 M $NaHCO_3$ stock solution, pH 8.0 to obtain a final concentration of 0.1 M. Check pH and adjust to 8.0 if necessary.
4. Incubate overnight at 2-8°C.
5. Filter on a gel e.g. Biogel P-30 in PBS with 0.1 M NaN_3 (final concentration).
6. Calculate final concentration of antibodies and store at 2-8°C. BSA or a similar protein should be added to a final concentration of 0.1% to stabilise the antibody during storage.

Cell separation
Using the Dynabeads immunomagnetic system, a target cell or molecule can be readily isolated using either a direct or an indirect technique (see Figure 17). With the ***direct technique***, a biotinylated antibody, specific for the target cell, is attached to the Dynabeads Streptavidin. This complex is then used to capture the desired target. The protocol is outlined below.

1. Before use, wash Dynabeads Streptavidin as specified in Chapter 5, section 1.1, page 156.

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